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PROJECT TITLE: Tobacco Physiology and Biochemistry
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I. LOW NICOTINE STUDY

A. **Objective**: To investigate the biochemistry of the nicotine biosynthetic pathway at putrescine N-methyltransferase (PMT) and specifically to isolate PMT from tobacco root extracts.

B. **Status**: Harvesting of the group 13 tobacco plants (50 plants, with an average root weight of 379 g) was completed, and the processing to the 40-65% ammonium sulfate stage of the root extracts from group 12 was done (1).

Various methods to concentrate the PMT-active samples were examined, for example, dialysis against polyvinylpyrrolidone (340,000 Daltons) or sucrose, and ammonium sulfate precipitation (65% saturation). All of the methods led to losses in PMT-activity and yield indicating the instability of the enzyme and or the fact that these methods of concentration are not suitable for our use (3,4).

Stability studies showed that propylene glycol (PG), ethylene glycol (EG) and glycerol (6) all maintained PMT-active samples at 4°C for up to 48 hr. Twenty percent EG did not maintain PMT-activity over a 24 hr period at 20°C (room temperature). The best pH condition for sample storage was found to be pH 8.0 (pH 5, 6, 7 and 8 tested) (6).

Further chromatographic studies were conducted with the phenyl-Sepharose column. Elution with 0.25 M NaCl appeared to be the most suitable condition to recover PMT activity when the column was equilibrated in the absence of EG or G. The incorporation of other washing steps (in the absence of a stabilizing agent) to precede the 0.25 M NaCl step resulted in much lower yields without significant increase in the specific activity (2,6). A 13-fold increase in specific activity of PMT over the ammonium sulfate stage was obtained by a sequential 1.5 M NaCl and 0.5 M NaCl step in the presence of 20% ethylene glycol with total recovery of PMT activity. This phenyl-Sepharose column was equilibrated in the presence of 1.5 M NaCl and 20% ethylene glycol (2,6).

Application of a sucrose concentrated sample (4-fold) to the Sephacryl gel filtration column was accomplished without decreasing the resolution of the column, i.e., the molecular weight of the peak PMT-active fraction remained the same (61,000 +/- 5000 Daltons) (4). The placement of this column as the first in the chromatographic series may alleviate down-stream processing and inherent enzyme losses.

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Further studies were conducted on characterizing PMT to insure that we are indeed isolating PMT. Five percent or less of the control PMT activities were observed for N-methylputrescine (NMP) and dipalmitoylphosphatidylethanolamine (DPPE) (5). NMP is a product of the PMT enzyme reaction and substrate for methyl-putrescine oxidase. DPPE is a membrane component of tobacco tissue and a physiological metabolite. Importantly, the Km for putrescine and S-adenosylmethionine were determined to be 8.0 X 10^{-4}M and 0.9 X 10^{-4}M, respectively, similar to literature values (5). These results indicate that PMT-like activity is being isolated.

Preliminary studies in which PMT active samples have been applied to native polyacrylamide gels indicate that we can recover PMT activity which is encouraging for the identification of PMT (3).

C. **Plans**: Extracts from the group 13 plants will be processed to the 40-65% ammonium sulfate stage. Modification of the conditions to elute PMT from phenyl-Sepharose in the presence of glycerol will be continued. Inhibitor studies will be examined for further characterizing PMT.

D. **References**:

1. Dunn, R. L. Notebook No. 7899.

2. Malik, V. Notebook No. 8542.

3. Sykes, A. Notebook No. 8526.

4. Yu, T. Notebook No. 8381.

5. Mooz, E. D. Notebook No. 8599.

6. Crockett, E. Notebook No. 8563.

II. ALTERNATE HUMECTANTS (PG/G-FREE SHEETS/CIGARETTES) (1)

A. **Objective**: To produce an acceptable cigarette (domestic Marlboro-type) which is PG/G-free by the end of the third quarter of 1988.

B. **Results**: Zero-time pad and material balance survivability tests on the production plant PG/G-free RL sheets were made in the Semi-works. Samples have been submitted for analytical evaluation. 100% test cigarettes made from production plant PG/G-free RCB and control RCB were subjectively reevaluated. The control and the PG/G-free test cigarette gave similar results, while the special control cigarette (containing no class tobacco) was considered to be atypical of RCB (2).

C. **Plans**: Zero-time pad and material balance survivability tests on the production plant, PG/G-free RCB sheets will be made in the Semi-works in August. Small-scale, control cigarettes and

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blended, PG/G-free cigarettes (containing PG/G-free flavors and casings) will be made in the Semi-works in August for subjective and toxicological evaluations.

D. References:

1. Mooz, E. D. Notebook No. 8599.

2. Ruziak, S. PG/G-free Program: 100% RL and RCB Cigarettes Made from production Plant Sheets. Memo to E. Mooz; 1988 July 8.

III. ALTERNATE HUMECTANTS (GLYCERINE-FREE SHEET/CIGARETTES) (1)

A. Objective: To produce an acceptable glycerine-free (G-free) cigarette (domestic Marlboro-type) for a POL test by the end of the third quarter of 1988.

B. Results: Control and G-free (POL #3582) cigarettes were remade in the Semi-works. Samples were submitted for analytical and subjective evaluation prior to being sent out. The Third Quarter Organizational Plans for the Alternate Humectant Program for 1988 have been issued (2).

C. Plans: Control and G-free cigarettes are scheduled to be sent out in mid-August.

D. References:

1. Mooz, E. D. Notebook No. 8599.

2. Mooz, Elizabeth D. Third Quarter Organizational Plans for the Alternate Humectant Program (AHP) for 1988: I. Production of a PG/G-free Cigarette and II. Production of a G-free, domestic Marlboro-type cigarette for a POL test. Memo to Dr. Robert McCuen. 1988 July 22.

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